Structures and Functions of C-type Lectins in Marine Invertebrates

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Structures and Functions of C-type Lectins in Marine Invertebrates

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Summary

Lectins distributing in all animal phyla form a diverse group of protein families that have in common the ability to recognize and bind certain carbohydrates. Although at least 13 animal lectin families are known to exist, many of marine invertebrate lectins are categorized in C-type lectin family, which was named from the Ca^{2+}-dependency for their carbohydrate binding activities. In contrast to a growing list of C-type lectins in marine invertebrates, their physiological roles are not fully understood. This review summarizes the structures and functions of marine invertebrate C-type lectins with our new findings.

Key words: C-type lectin, lectin, marine invertebrate

Marine biodiversity is extremely high as a consequence of the extraordinary variability of the marine biosphere. Marine invertebrates live in areas rich in food particles with an abundant microflora that act as a potential source of infection. Although invertebrates lack inducible immunoglobulins, they seem to have evolved effective means for self and non-self recognition to clear invaded microbes. Glycoconjugates are involved in binding bacteria to macrophages, and it is conceivable that glycoconjugate determinants also function in invertebrate phagocytosis. Lectins are carbohydrate-binding proteins other than immunoglobulins that agglutinate cells and/or precipitate glycoconjugates through interaction with glycoproteins or glycolipids. Therefore, they have been recognized as recognition molecules. Lectins are found in all kingdoms of living organisms ranging from virus through bacteria and plants to animals, and play important roles in different biological processes such as cell-cell adhesions, cell communications, signaling events, nutritional effects, cytotoxicity, and infection by viruses, etc as counterparts of carbohydrates. Invertebrate lectins have been isolated from diverse sources, in many cases as multimeric isoforms. With the number of

antigens that invertebrate encounter, the presence of multiple lectins with heterologous binding sites is certainly advantageous.

Animal lectins have been classified on a structural basis into at least 13 animal lectin families, including C-type lectins and galectins, which are classic major families (Kilpatrick, 2002). The carbohydrate recognition domain (CRD) of C-type lectins, of 115–150 amino acid residues, requires Ca^{2+} for carbohydrate binding activities. The CRD structure has a characteristic double-loop (loop-in-a-loop) stabilized by two highly conserved disulfide bonds located at the bases of the loops, as well as a set of conserved hydrophobic and polar interactions. Since Noguchi reported the presence of a lectin (hemagglutinin) from American lobster, *Homarus americanus* in 1903, a number of invertebrate lectins have been isolated and characterized. Most of them, however, belong to C-type lectin family as shown in Table 1. This review highlights current knowledge concerning marine invertebrate lectins with particular emphasis on their structures and functions.

Marine Invertebrate C-type Lectins on a Phylogenetic Basis

Practically all classes and subclasses of marine invertebrates examined have lectins. Lectins are present mainly in the hemolymph and reproductive organs, e.g., albumin glands, eggs, and also occur on membranes of hemocytes that function in innate immunity (Vasta, 1992). The number of marine invertebrate lectins, of which amino acid sequences have been determined either by direct sequencing or cDNA sequencing, is still growing.

Protozoa

The phylum Prorifera is the oldest group of multicellular animals. The phenomenon of the species-specific reaggregation of mechanically or enzymatically dissociated sponge cells has attracted many investigators, because it provides a very useful model for studying molecular mechanisms of selective cell-cell adhesion and intercellular recognition processes believed to play important roles in embryonic tissue formation. The model that extracellular aggregation factors and aggregation receptors on cell surface are linked by lectins is now assumed as a major step forward in understanding the complex process of aggregation in sponge cells (Wagner-Hulsmann et al., 1996).

Aggregation factor isolated from *Aphrocallistes vastrus* turned out to be a 34 kDa glycoprotein with a 24 kDa proteinous core whose function was inhibited by D-galactose (Gal) but not D-mannose (Man) (Müller et al., 1984). Two highly similar C-type lectins, named LECC1 and LECC2, were cloned from hexactinellid sponge, *Aphrocallistes vastus* (Gundacker, et al., 2001). Other types of lectin family have been also isolated from *Geodia cydonium* (Jarchow and Burger, 1998) and *Axinella polypoides* (Buck et al., 1998). These studies indicate that during

Table 1. Distribution of C-type lectins in marine animals

Species	Lectin	Lengh (amino acid residues)	Tissue	Specificity
[Protozoa]				
Sponge				
Aphrocallisteres vastus	LECC1 LECC2	191	—	Gal
[Mollusca]				
Abalone				
Haliotis laevigata	Perlucin (PLC)	155	Nacre	Gal/Man
[Arthropoda]				
Acorn barnacle				
Megabalanus rosa	BRA-1, -2, -3	173/138	Hemolymph	Gal
Balanus rostratus	BRL	182	Hemolymph	Gal
[Echinodermata]				
Sea cucumber				
Cucumaria echinata	CEL-1, and -IV	140/157	—	GalNAc
Stichopus japonicus	SJL-I	143	—	Gal
Sea urchin				
Anthocidaris carssispina	ECH	147	Hemolymph	Gal/ Galβ1-3GalNAc
Starfish				
Asterina pectinifera		168	—	GalNAc
[Protochordata]				
Tunicate				
Polyandrocarpa misakiensis	TC14-1, 14-2	125		Gal
Halocynthia roretzi	—	327	—	Gal
[Vertebrata]				
Fish				
Carcharchinus springeri	—	166	Cartilage	—
Anguilla japonica	AJL-2	142	Skin	Lac
Oncorhynchus mykiss	TCL-1	238	Serum	Gal/Man

the evolution of unicellular eukaryotes to multicellular animals, lectins and their receptors played a fundamental role.

Mollusca

The molluscs have probably been investigated for the involvement of lectins in a defense reaction more than any other groups of invertebrates. A matrix

protein, named perlucin, was isolated from nacreous layer of the shell of abalone (*Haliotis laevigata*) (Weiss et al., 2000). Perlucin (*Mr* 17 K) consists of 155 amino acid residues including a glycosylated asparagine. The sequence of the first 130 amino acids shows a high similarity to the C-type lectin domain especially asialoglycoprotein receptors. Although the sequence motif, Gln-Pro-Asp at residues 92–94 in perlucin, suggested its specificity for Gal, the binding assay showed divalent metal ion-dependent binding to (neo) glycoproteins, and the ability to distinguish between Gal and Man was rather low (Mann et al., 2000). Moreover, perlucin nucleates calcium carbonate crystals and is incorporated into the crystals (Blank et al., 2003).

Crustacea

The horseshoe crabs were one of the first sources of animal lectins to be discovered. Five hemocyte lectins (tachylectins) and two plasma lectins of the Japanese horseshoe crab (*Tachypleus tridentatus*) have been isolated and characterized (Inamori et al., 1999 ; Chen et al., 2001).

The multiple C-type lectins, named BRA-1, BRA-2, and BRA-3, were isolated from the hemolymph of the acorn barnacle, *Megabalanus rosa* (Kamiya and Ogata, 1982). They have apparent Gal binding activities as well as inhibitory activity toward the crystal growth of calcium carbonate (Muramoto et al., 1994a). BRA-3 (*Mr* 64 K) is composed of four identical subunits (*Mr* 16 K) which consist of 138 amino acid residues with no glycosylation site (Muramoto and Kamiya, 1986). Both BRA-1 (*Mr* 330 K) and BRA-2 (*Mr* 140 K), on the other hand, are composed of identical subunits (22 kDa), which consist of 173 amino acid residues with one *N*-glycosylation site at Asn-39 in N-terminal region (Muramoto et al., 1990a and 1990b). BRA-2 decreased the protease resistance, structural stability, and various activities such as hemagglutination and modulation of calcium carbonate crystallization significantly by deglycosylation (Matsubara et al., 2005). The amino acid sequences of BRA-1/-2 and BRA-3 show only 16% identity.

BRL (*Mr* 120 K) whose subunit (35 kDa) consists of 182 amino acid residues from *Balanus rostratus* hemolymph is a multimeric protein (Toda et al., 1998). BRL has no inhibitory activity toward the crystal growth of calcium carbonate. BRL was 46% identical to BRA-2 and 15% identical to BRA-3 (Muramoto et al., 2001). Frontal affinity chromatography method using an oligosaccharide library demonstrated that the multiple C-type lectins had distinct carbohydrate binding specificities ; that is, BRA-2 could strictly distinguish α2, 6 Neu5Ac from α2, 3 Neu5Ac, BRA-3 recognized the oligosaccharides containing terminal α-linked Gal, and BRL could recognize antigenic epitope (Lewis^x or Lewis^a-epitope) (unpublished data).

Echinodermata

The echnoderms are regarded as among the most advanced invertebrates in evolutionary terms rather close phylogenetically to tunicates and vertebrates. Four Ca^{2+}-dependent and Gal/*N*-acetyl-*D*-galactosamine (GalNAc)-specific lectins (CEL-I, -II, -III, and -IV) have been isolated from body fluid of sea cucumber, *Cucumaria echinata*. CEL-I and CEL-IV belong to the C-type lectin family (Hatakeyama et al., 2002). CEL-I (32 kDa) is composed of two identical subunits of 140 amino acid residues cross-linked by a single disulfide bond, while CEL-IV comprises 157 amino acid residues with a molecular mass of 17 kDa without a disulfide bond. They have only 30% sequence identity, each other. CEL-I is homologous to the C-type lectin, echinoidin, from a sea urchin (*Anthocidaris crassispina*) with 36% identity (Giga et al., 1987). CEL-IV shows 40% identity to SJL-I, from a sea cucumber (*Stichopus japonicus*), and 33% identy to echinoidin. Recently, CEL-I was expressed in *E. coli* and crystallized for X-ray structural analysis (Hatakeyama et al., 2004).

Protochordata

Since tunicates occupy a pivotal intermediary position between invertebrates and vertebrates, they are of interest as a means to search for ancestral characteristics of vertebrates that were established before their divergence from invertebrates. A tunicate C-type lectin of 14 kDa (TC14-1) whose subunit consists of a total of 147 amino acid residues has been isolated from the budding animal, *Polyandrocarpa misakiensis*. It consists of a single carbohydrate recognition domain that binds to Gal and L-fucose (Fuc) (Suzuki et al., 1990). TC14-1 is the first dimeric C-type lectin of which a three-dimensional structure has been reported (Poget et al., 1999).

A galactose-specific lectin from the plasma of the ascidian *Halocynthia roretzi* consists of an N-terminal repeated sequence, a fibrinogen-related sequence and a sequence homologous to the C-type lectin family (Abe et al., 1999). The lectin is secreted from the hepatopancreas and can enhance phagocytosis by hemocytes, suggesting its involvement in defense mechanisms in *H. roretzi*. The discovery of an ascidian mannan binding lectin-associated serine protease and an ascidian homologue of mammalian complement component C3 highlights its pivotal role in host defense against infection (Ji et al., 1997). More recently, a putative C-type lectin has been cloned from *Botryllus schlosseri* which possesses an immunoglobulin domain as well as a CRD (Pancer et al., 1997).

CRD Structures of C-type Lectins

Folds and Genes

The amino acid sequences of C-type lectin domains from marine invertebrates

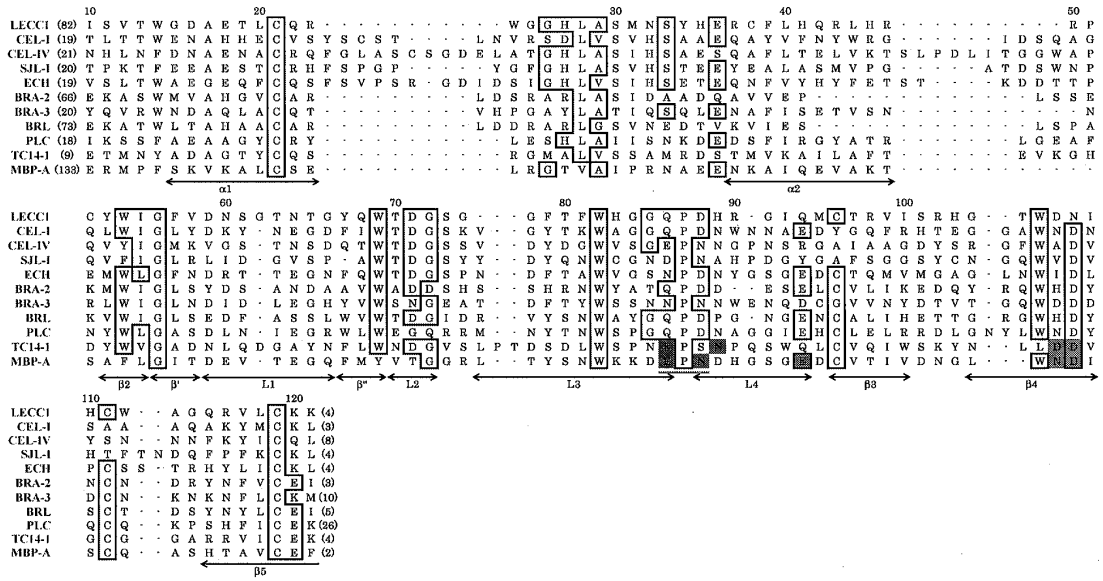

FIG. 1. Structural sequence alignment of C-type lectin from marine invertebrates (A). Top numbers show the amino acid residues in tunicate (*Polyandrocarpa misaliensis*) C-type lectin (TC14-1). The number in bracket show the amino acid residues which is abbreviated for this alignment. Residues of carbohydrate and calcium ion binding site (for TC14-1 and MBP-A) are shared in grey. The elements it secondary structure shown refer to TC14-1. Amino acid residues identical to those of check for other identical residues were boxed. Provide references for sequences or use following statement: The amino acid sequences were taken from the references found in the text.

are aligned in Fig. 1. The sequence homologies among them are 12–48%. CRD of C-type lectins adopts a typical fold; one half of the molecule consists of a long two stranded β-sheet and two α-helices, while the second half contains the calcium and carbohydrate-binding site, and is mostly formed of non-repetitive loop structures. This fold is conserved in all the known C-type lectin structures, including the human and rat serum mannose-binding protein (Sheriff et al., 1994; Weis et al., 1991), and the tunicate lectin, TC14-1 (Poget et al., 1999). The three dimensional structure of BRA-2 was predicted by using the structure of lithostathine as a template (Fig. 2). The short β-strand $\beta 2$ acts as a connection between the two β-sheets formed by the N- and C-terminal strands $\beta 1$ and $\beta 5$, and the $\beta 3$ and $\beta 4$ strands. The extended loops 3 and 4 are involved in sugar binding together with strand $\beta 4$. BRA-2 and BRA-3 have covalent homodimer structures cross-linked by two disulfide bonds at the N-terminal and the C-terminal region, respectively, while the TC14-1 subunit forms a non-covalent dimer by both intermolecular hydrogen bonds and hydrophobic interactions at the N- and C-terminal regions. The number and mode of inter-subunit disulfide bonds vary among C-type lectins.

For the C-type lectins whose gene structures have already been determined,

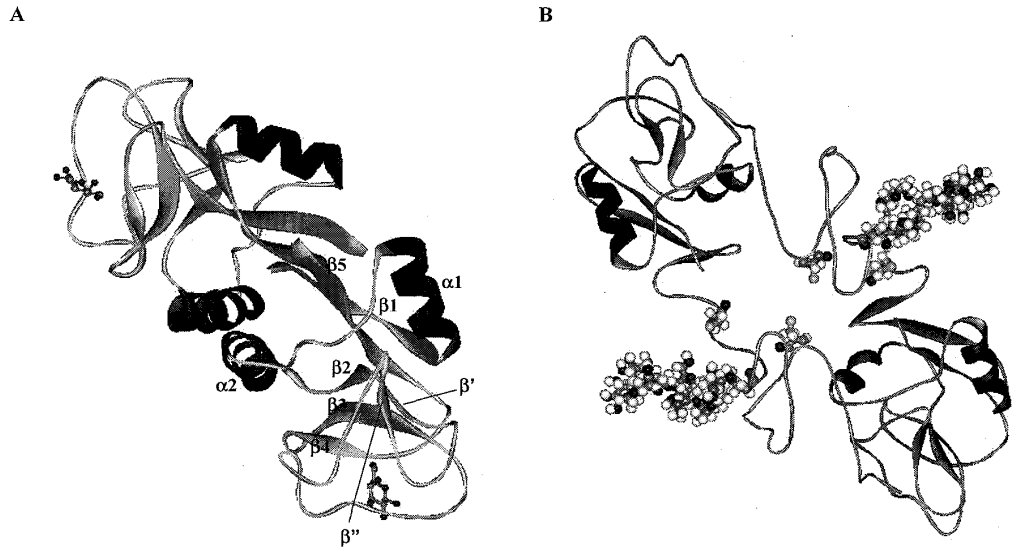

FIG. 2. 3-D molecular structural model. (A) The crystal structural model of TC-14-1 with galactose (PDB ID; 1TLG); (B) Putative structural model of BRA-2. Homology modeling was performed with a software tool, the molecular operating environment (MOE) system (Chemical computing Group Inc., Canada). Lithostathine (human pancreatic stone), PDB ID; 1QDD, was selected as a template.

they can be categorized into two groups on the basis of the exon/intron structure of the CRD (Benzouska et al., 1991). In the first group, the CRD is encoded by three separate exons. The group includes the rat asialoglycoprotein receptor, the core protein of chicken cartilage proteoglycan, and the Kupffer cell receptor. In contrast, the CRDs of rat and human mannose-binding proteins, and human pulmonary surfactant apoprotein are encoded by single exons. In the case of the acorn barnacle lectins, the BRA-3 CRD is encoded by three exons, whereas the BRA-2 CRD is encoded by a single exon (Takamatsu et al., 1994). The presence of the two distinct gene structures in C-type lectin family gave an insight into the events in the molecular evolution of the CRDs; i.e., duplication and insertion or deletion within a primordial CRD gene generated two types of precursor CRD genes containing or lacking introns. Through exon shuffling, these precursor genes became associated with various functional domains to generate present-day multifunctional C-type lectins existing in mammals. These events must have led to distinct carbohydrate-binding specificities as well as divergent biological functions.

When searching for C-type lectin-like domains in the nematode (*Caenorhabditis elegans*) genome, about 180 sequences were found (Dodd and Drickamer, 2001). With about 20,000 open reading frames in the genome, the lectin domain is thus ranked seventh in occurrence. Interestingly, the domain does not exist in the genome of unicellular organisms (yeast), indicating that it emerged in

Metazoa. Despite the abundance of C-type lectin-like domains in the invertebrate genomes, the domain architectures do not match those of the vertebrate groups. This finding suggests that simple extrapolation from vertebrate lectins to invertebrate lectins cannot be achieved *vice versa*.

Carbohydrate and Calcium Binding Sites

X-ray structure analysis of TC14-1 demonstrated that the carbohydrate binding occurred indirectly through a calcium ion (Poget et al., 1999). There is only one calcium binding site per domain in the TC14-1 structure. Side-chain oxygen atoms of Glu-86, Asn-89, Asp-107 and Asp-108 as well as the main-chain carbonyl oxygen of Asp-108 are involved in the binding (Fig. 1). The last two sites to reach pentagonal bipyramidal coordination are occupied by the galactose 3 and 4-hydroxyl oxygen atoms (Poget et al., 1999). Solvent molecules play a role in the structure of the complex by mediating indirect protein-carbohydrate interactions that are bridged by a single water atom. The Gal selectivity of TC14-1 observed in the binding studies can be managed by the distribution of hydrogen-bond acceptors and donors around the sugar hydroxyl groups 3 and 4 and the presence of a Trp-100 side-chain close to the binding site. A stabilizing effect of bound Ca^{2+} on CRD structure has been reported for a number of C-type lectins. Ca^{2+} removal greatly increases the susceptibility to proteolysis and changes properties of the CRD. However, NMR studies of the TC14-1 have shown that its loops maintain its compact fold when Ca^{2+} is removed (Poget et al., 1999).

The CRD of mannose binding protein (MBP) has two binding sites for calcium ions (Kolatkar and Weis, 1996). The residues involved in the first binding site are Asp-161 (60 in TC14-1), Glu165 (64 in TC14-1), Asp-188 (89 in TC14-1) and Asp-194 (95 in TC14-1). The second binding site is formed by Glu-185 (86 in TC14-1), Asn-187 (89 in TC14-1), Glu-193 (94 in TC14-1), Asn-205 (107 in TC14-1) and Asp-206 (108 in TC14-1) which also constitute the carbohydrate binding site. The positions 185 (86 in TC14-1) and 187 (89 in TC14-1) have been shown to be a major determinant of ligand binding specificity in the CRD based on experiments with mutated proteins and sequence comparisons of CRDs with known carbohydrate-binding specificity, where the two mutations Glu-185 (86 in TC14-1)-Gln and Asn-187 (89 in TC14-1)-Asp induced weak preferential galactose binding (Iobst and Drickamer, 1994; Kolatkar and Weis, 1996). The carbohydrate binding sites of C-type lectins were classified as Gal-type or Man-type according to the presence of the sequence motif Gln-Pro-Asp (QPD) or Glu-Pro-Asn (EPN), respectively. In all of invertebrate C-type lectins, they show the affinity toward Gal or GalNAc as its derivative. However, perlucin bound both types of sugars (Gal and Man) with very similar affinities, indicating that not all factors responsible for carbohydrate recognition are known and that

it may therefore be difficult to predict binding selectivity by sequence comparisons and homology modeling. It is clear that CRDs with their largely conserved structural framework can interact in multiple ways with a carbohydrate ligand, and this is a distinctive feature not shared by another animal lectin family, such as galectins.

Biological Functions of C-type Lectins in Marine Invertebrates

Lectins in Biodefense System

Mammalian lectins (mannose binding proteins) have been demonstrated to play an important role in host-pathogen interactions by specific recognition with cell surface substances of bacteria (Weis et al., 1998). Studies of a number of invertebrate species, which do not express immunoglobulin antibodies, indicate that lectin-mediated complement pathways evolved before antibody-based complement activation mechanisms (Vasta et al., 1999). Evolutionary homologs of C3 have been identified in both sea urchin and tunicates, which share common ancestors with vertebrates.

Immunity to infectious agents is mediated by two general systems, innate and acquired. Acquired immunity, found only in vertebrates, is one function of B and T lymphocytes, which produce an infinite multitude of specific antigen receptors and antibodies through somatic gene rearrangement. On the other hand, innate immunity is phylogenetically older than acquired immunity, and a certain form of innate immunity is present in all multicellular organisms. Non-self-recognizing proteins involved in innate immunity seem to recognize mainly carbohydrate moieties on pathogens. Furthermore, the innate immunity in invertebrates is also triggered by polysaccharides, such as lipopolysaccharides (LPS) of Gram-negative bacteria, lipoteichoic acid of Gram-positive bacteria, glycolipids of mycobacterium and mannans of yeast.

In *Megabalanus rosa* multiple C-type lectins (BRA-1, BRA-2 and BRA-3) are major proteins in the coelomic fluid; i.e. 20–30% of the total proteins (some individual contains over 60%), and show the seasonal dynamics as related to reproductive cycle (Muramoto et al., 1994b). These facts suggest that BRAs participate in not only humoral immunity related to biological defense but also in other biological functions. In addition to the opsonin activity (Yamazaki et al., 1983), BRAs may play important roles in a complex and neat cellular and humoral defense system in the barnacle (Kamiya et al., 2002). The lectins on hemocytes are supposed to be involved in repairing the shell breakage of *M. rosa*.

Lectins in Biomineralization

Calcified hard tissues and skeletons, such as various shells and pearls, provide structural support and protection for many marine invertebrate phyla, including

Mollusca, Crustacea and Echinodermata. A calcified shell layer is composed of two polymorphs of calcium carbonate, aragonite or calcite and an organic matrix. The organic matrix associated with shells is thought to be essential for shell formation (biomineralization) by determining structural properties such as crystal type, size and shape (Gunthorpe et al., 1990).

The apparently widespread occurrence of C-type lectin-like domain proteins in calcium carbonate biominerals was realized only very recently. Such proteins have been isolated not only from hard tissues of invertebrates (Mann et al., 2000) but also from vertebrates (Mann et al., 1999 ; Reggi et al., 2001 ; Zhou et al., 2001 ; Lakshminarayanan et al., 2003). The first example was pancreatic stone protein (DeCaro et al., 1988). This protein, also known as lithostathine, was isolated as a proteolytically modified form from human pancreatic stones. Lithostathine is believed to bind calcium carbonate and prevent calcium carbonate from precipitation and forming calcitic pancreatic stones. Another example is the avian eggshell protein ovocleidin (Mann and Siedler, 1999). Several proteins apparently containing C-type lectin-like sequences, but lacking the conserved amino acids necessary for calcium ion-dependent carbohydrate binding, were detected in the organic matrix of sea urchin embryonic spicules (Harkey et al., 1995 ; Killian and Wilt, 1996).

The proteins that comprise the integral spicule matrix of a sea urchin (*Strongylocentrotus purpuratus*) embryo are postulated to interact with specific faces of the calcite crystal when occluded within the mineral and control the spicule growth (Wilt et al., 2003). The proteins named SM30, SM37 (PM27) and SM50 are similar to the CRD domain of C-type lectins (Harkey et al., 1995 ; Killian and Wilt, 1996).

A protein, named perlucin with M_r 17,000, was isolated from the shell of the mollusc *Halotis laevigata* (abalone) after demineralization of the shell. Perlucin belonged to a heterogeneous group of proteins consisting of a single C-type lectin domain (Mann et al., 2000). Perlucin increased the precipitation of calcium carbonate from a saturated solution, indicating that it may promote the nucleation and/or the growth of calcium carbonate crystals. This result indicates that perlucin may nucleate and bind calcium carbonate crystals and may serve to connect the chitin layer and the aragonite layer.

The multiple C-type lectins (BRAs) in an acorn barnacle inhibit the crystal growth of supersaturated calcium carbonate solution at less than 33 μg/mL. BRAs affect the size and morphology of the calcium carbonate crystals (Muramoto et al., 2001). The inhibitory activities dramatically change by altering the conformation of BRAs (Muramoto et al., 1994a) and by immobilizing BRAs onto a chitosan coupon (Kamiya et al., 2002).

Lectins in Other Functions

Ehinoidin from sea urchin has an Arg-Gly-Asp (RGD) sequence which is known to be an active signal in cell adhesive molecules including fibronectin, vitronectin and the von Willebrand factor (Giga et al., 1987). The cell adhesion activity of echinoidin was mediated by the RGD sequence within the lectin, but not by the carbohydrate recognition domain (Ozeki et al., 1991). Moreover, C-type lectins (TC14 families) from tunicate (*Polyandrocarpa misakiensis*) are involved in a variety of biological functions, such as cytostatic role in regulating cell growth, cell adhesion and cell differentiation during asexual reproduction (Matsumoto et al., 2001). The Gal binding site of TC14-1 is essential for this activity.

Conclusion

The past few years of invertebrate lectin research have provided us with a growing volume of information about molecular structure, gene organization, evolutionary relationships and biological roles. The recent knowledge concerning the structures and functions of C-type lectins in marine invertebrate is reviewed here. Although a number of C-type lectins have been isolated and characterized from marine invertebrate, they have been also obtained from fish recently (Tasumi et al., 2002 ; Achenbach and Ewart, 2002 ; Hosono et al., 2005). Type II antifreeze protein is one of fish-specific C-type lectins. This protein binds to ice in a Ca^{2+}-dependent manner (Ewart et al., 1998). Additionally, another type of lectins from marine life is increasing with development in experimental technique ; e.g., tachylectin (Kawabata, 2002), a novel lectin from sea cucumber (CEL-III) (Nakano et al., 1999), rhamnose-binding lectin (Tateno et al., 1998).

We have proposed that the multiple C-type lectins (BRAs) from the hemolymph of the acorn barnacle, *Megabalanus rosa*, participate, not in a single role, but in multiple roles, which include biomineralization and biodefense.

Future progress of the study on marine invertebrate lectins will elucidate the nature of the forces that mediate the protein-carbohydrate interactions, their kinetics, and the structural changes produced in the lectin upon binding to ligand, and the identification and characterization of the biologically relevant ligands. It will provide solid support for the definitive understanding of the biological roles of marine invertebrate lectins. Furthermore, the investigation for novel lectins from marine invertebrates will contribute to developing new tools for research in glycomics or nanotechnology.

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